

#2147

THE COUNCIL FOR TOBACCO RESEARCH

110 EAST 59TH STREET  
NEW YORK, N. Y. 10022  
(212) 421-8885

AUG 5 1974

Application for Research Grant

Date: 7/30/74

(Use extra pages as needed)

1. Principal Investigator (give title and degrees):

Una S. Ryan, Ph.D., Senior Scientist  
Papanicolaou Cancer Research Institute  
Assistant Professor of Medicine, University of Miami School of Medicine

2. Institution & address:

Papanicolaou Cancer Research Institute  
1155 N.W. 14th Street  
Miami, Florida 33136

Mailing Address: P.O. Box 6188  
Miami, Fla. 33123

3. Department(s) where research will be done or collaboration provided:

Cardiopulmonary Unit  
1425 N.W. 10th Avenue  
Miami, Florida 33136

4. Short title of study:

ENDOCRINE FUNCTIONS OF THE LUNGS

5. Proposed starting date: January 1, 1975

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

There are two main aims of this research proposal: the first is to obtain direct evidence on the precise subcellular site of pulmonary angiotensin converting enzyme and to relate these data to understanding of the non-ventilatory functions of the lung. The second aim is to develop rapid, simple and sensitive assays of pulmonary angiotensin converting enzyme to facilitate future studies of the effects of physiologic changes and environmental influences (e.g. tobacco smoke) on the ability of the lungs to convert angiotensin I to its potent lower homolog, angiotensin II.

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8. Brief statement of working hypothesis:

2.

A. It is now well recognized that the lungs process the circulating vasoactive polypeptides, bradykinin and angiotensin I, the former being inactivated while the latter is activated. Previously, we have postulated that the enzymes responsible for the metabolism of these polypeptides are situated on or close to the luminal surface of pulmonary capillary endothelial cells. Studies from our laboratory over the past six years, as well as from others, have supported the hypothesis but have not proved it. Within the last year, it has been shown that the enzyme that activates angiotensin I (by conversion to angiotensin II) is also responsible for the inactivation of bradykinin. We have raised antibodies to this enzyme and now propose to use the antibodies, labelled for immunocytochemistry, to provide a definitive test of our hypothesis.

B. Our previous studies on the metabolism of the vasoactive polypeptide hormones required intrinsically labelled synthetic hormones. The hormones and their radioactive metabolites were identified after lung perfusion by chromatographic, electrophoretic and countercurrent distribution techniques requiring long periods of time. Whereas this approach was essential for defining the biochemical mechanisms by which the hormones were metabolized, it is evident that this approach does not yield itself to studies of instantaneous changes in pulmonary function that may occur as a result of subtle influences such as tobacco smoking. Therefore, in addition to testing the hypothesis described above, we would like to undertake a technical program to develop inexpensive, radioactive substrates which will lend themselves to the rapid, simple and sensitive measurement of the turnover rate of angiotensin converting enzyme. Such substrates will be of immediate use in investigations of the ways in which the lungs can modulate their "endocrine" functions in response to physiological stimuli and to inhalants such as tobacco smoke.

9. Details of experimental design and procedures (append extra pages as necessary)

Appended. See section beginning 9-1.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The investigators of this program constitute the Cardiopulmonary Unit of the Papanicolaou Cancer Research Institute. These laboratories occupy the Sieron Building, situated within the University of Miami Medical Center. Our laboratories include about 5,000 square feet of floor space, and include a cold room, a secretarial area, vivarium, and rooms for electron microscopy, biochemistry and polypeptide synthesis.

Our laboratories have access to the full facilities of the Papanicolaou Institute and of the Department of Medicine, University of Miami. We also have free access to the full library facilities of the University of Miami Medical Center.

Equipment

Philips EM 301 electron microscope  
LKB Ultratome III  
LKB knife maker  
Durst enlarger and ancillary photographic equipment  
Zeiss binocular microscope with camera and phase contrast attachments  
Wild dissecting microscope  
Gilson and LKB fraction collectors  
Craig-Post 200-transfer countercurrent  
Spinco 120C amino acid analyzer  
Unilux II liquid scintillation counter  
Actigraph strip counter  
Buchi flash evaporator

Two-channel autoanalyzer  
VirTis lyophilizer  
Apparatus for manual and automatic synthesis of polypeptides  
Circulating water baths and incubators  
Pumps for infusion and respiration of small animals  
Apparatus for thin-layer chromatography and electrophoresis  
Mettler top pan balance  
Radiometer pH meters (2)  
Radiometer automatic titrator  
Grass four-channel polygraph

11. Additional facilities required:

No new facilities are required for the purposes of this grant.

12. Biographical sketches of investigator(s) and other professional personnel (append):

Appended

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Appended

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## 14. First year budget:

## A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)  
even if no salary requested):

% time

Amount

\*Una Ryan, Ph.D. Principal Investigator  
 \*\*J.W. Ryan, M.D., D.Phil. Co-Investigator  
 \*\*A. Chiu, Ph.D. Co-Investigator  
 Fringe Benefits

REDACTED

## Technical

Debra Hodges  
 Erica Clements

Lab Tech  
 Lab Tech

REDACTED

Sub-Total for A 26,383

## B. Consumable supplies (by major categories)

Animals: rats \$400; rabbits, \$200. Golden hamsters, \$100 700  
 Photographic supplies 1,000  
 Chemicals and glassware, general 800  
 Fixatives, embedding and staining materials 700  
 Microperoxidase, 250 mg 600  
 Reagents for peptide synthesis and conjugation techniques 700

Sub-Total for B 4,500

## C. Other expenses (itemize)

Travel (1 trip per investigator @ \$500 each) 1,500  
 Shipping charges 150  
 Reference Books 75  
 Regrinding diamond knives for microtomy 350

Sub-Total for C 2,075

Running Total of A + B + C 32,958

## D. Permanent equipment (itemize)

-0-

Sub-Total for D -0-

## E. Indirect costs (35% of A+B+C)

E 4,944

Total request 37,902

## 15. Estimated future requirements:

|        | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total  |
|--------|----------|-------------------|----------------|------------------|----------------|--------|
| Year 2 | 29,021   | 4,770             | 2,075          | -0-              | 5,380          | 41,246 |
| Year 3 | 31,923   | 5,056             | 2,075          | -0-              | 5,858          | 44,912 |

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## BUDGET JUSTIFICATION

\*Una Ryan. The major portion of Dr. Una Ryan's salary is paid by the American Heart Association through her tenure of an Established Investigatorship. The salary requested here is calculated as 10% of the allowed supplement.

\*\*James W. Ryan and Andrew Chiu. Salary funds are requested in proportion to per cent times to be spent on this research project. Dr. Chiu's biographical sketch is attached.

Fringe benefits are calculated at 15% of salaries and include social security, health insurance, life insurance, unemployment and pension plan.

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project                                      | Source<br>(give grant numbers)                       | Amount | Inclusive<br>Dates |
|---|--|--------|--------------------|
| Endothelium: Structure and Functions<br>(salary only) | American Heart Association<br>(#72 160)              | 16,000 | 7/1/74 - 6/30/75   |
| *Fine Structure of Normal Lung Cells <u>in Situ</u>   | National Heart and Lung Institute<br>(#N01-HR3-3015) | 45,991 | 10/1/74 - 9/30/75  |

\*The work solicited by the contract of the National Heart and Lung Institute does not overlap in approach, concept or timing with the research program described in the present application.

PENDING OR PLANNED

| Title of Project | Source<br>(give grant numbers) | Amount | Inclusive<br>Dates |
|------------------|--------------------------------|--------|--------------------|
|                  |                                |        |                    |
|                  |                                |        |                    |
|                  |                                |        |                    |
|                  |                                |        |                    |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

Panico laou Cancer Research Institute

Mailing address for checks

P.O. Box 6188  
Miami, Florida 33123

Principal investigator

Typed Name Una S. Ryan  
Signature Una S. Ryan Date 8/1/74  
Telephone 305 324-4341  
Area Code Number Extension

Responsible officer of institution

Typed Name Dr. Julius Schultz  
Title Director and President  
Signature Julius Schultz Date 8/2/74  
Telephone 305 324-5572 34  
Area Code Number Extension

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## 9. Details of experimental design and procedures

### 1. Final approach to enzyme localization by immunocytochemistry

#### Background

Over the past six years, a large body of indirect evidence has been presented indicating that a variety of polypeptide hormones, prostaglandins, nucleotides and amines disappear during circulation through the lungs (see Vane, 1969, for review). Some six years ago, we began a series of experiments to examine the mechanisms by which these phenomena occur. In brief, evidence at hand indicates that circulating adenine nucleotides, angiotensin I and some of the kinins are metabolized by hydrolase enzymes on or near the luminal surface of pulmonary endothelial cells (Ryan et al., 1968, 1970a and b, 1971, 1972; Ryan and Smith, 1971a and b, 1973; Smith and Ryan, 1969, 1970, 1971 a and b, 1972, 1973). By contrast, prostaglandins of the E and F series appear to be removed from the circulation by cellular uptake.

From the beginning of our experiments, it was evident that we had found an entirely new mechanism of hormone metabolism (Ryan et al., 1968). Firstly, blood enzymes are not required. Secondly, the lungs do not secrete hydrolase enzymes having the specificity required to degrade angiotensin I, bradykinin or adenine nucleotides. Thirdly, lungs perfused with radioactive angiotensin I, bradykinin or nucleotides do not retain radioactivity. On these points alone, we began to suspect that the requisite hydrolase enzymes exist on or very near the luminal surface of pulmonary endothelial cells. The concept was further supported by examination of the kinetics of disappearance of intrinsically-labelled parent compounds. We found that the mean transit times and volumes of distribution of radioactivities are no greater than those of blue dextran (MW > 2,000,000), a substance unlikely to leave the vascular space. Using radioactive bradykinin, adenine nucleotides or angiotensin I, there is a quantitative recovery of radioactivity in the pulmonary venous effluent; yet little, if any of the radioactivity remains in the form of the parent compound.

In parallel studies, we began investigations of the morphology of pulmonary endothelial cells, placing special emphasis on the occurrence of fine structure of the plasmalemma that might explain the phenomena described above. It was evident in the first studies that pulmonary endothelial cells, especially those of capillaries, are densely packed with caveolae (pinocytotic vesicles), most of which communicate with the exterior of the cell through small stomata. It was also evident that the caveolae along the luminal surface communicate directly with circulating blood and have the effects of expanding the surface area of the cell itself and of increasing the volume of the vascular lumen (Smith and Ryan, 1970).

Endothelial caveolae became of even greater interest as a consequence of our finding that the caveolae contain ATPase and 5'-nucleotidase, enzymes apparently not shared by the remainder of the plasma membrane (Smith and Ryan, 1970, 1971). Recently, we have shown by combined biochemical and cytochemical techniques that 5'-AMP entering the pulmonary artery is, in fact, dephosphorylated by enzymes of the caveolae open to the vascular lumen (Ryan and Smith, 1971b).

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Whereas the data on the endothelial processing of adenine nucleotides is direct, evidence on the processing of angiotensin I and bradykinin is still, to an extent, inferential. Specific cytochemical tests are not available for polypeptide hormones. Nonetheless, our hypothesis on the means of metabolism of angiotensin I and bradykinin has been tested by a variety of techniques. Firstly, one implication of our hypothesis is that angiotensin I and bradykinin should be metabolized to characteristic products by the plasma membrane fraction of whole lung homogenates. We have found that the lung plasma membrane fraction does, in fact, convert angiotensin I to angiotensin II and reduces  $^{14}\text{C}$ -Pro<sup>2</sup>-bradykinin to radioactive Prolyl-Proline (Ryan and Smith, 1971a). Secondly, isolated pulmonary endothelial cells should, on incubation with the parent peptides, produce the same products. This has been found to be the case (Ryan and Smith, 1973; Smith and Ryan, 1973).

Having adduced evidence that the membranes of endothelial caveolae have metabolic properties apparently not shared by the remainder of the plasma-lemma, we have begun to examine caveolae for ultrastructural specializations. Data obtained so far indicate that caveolae have a circular skeletal structure, possibly functioning to maintain the patency of the stomata and the integrity of the stomal diaphragm (Smith and Ryan, 1972). In studies of thin sections, it has also become apparent that the inner, concave surface of the caveola membrane contains regularly-spaced globular structures, possibly enzymes or binding sites (Smith and Ryan, 1972, 1973; Smith et al., 1973). Our freeze-fracture studies have confirmed and extended these findings. Of particular interest, intramembranous particles (75-100 Å in diameter) are prominent in association with caveolae. The size of these particles, some of which are thought to be enzymes, is about that expected for a globular enzyme having a molecular weight of 150,000-300,000. This point may be in agreement with the findings of Dorer et al. (1972), which show that the angiotensin I converting enzyme of hog lung has a molecular weight of 280,000. Our recent experience with negatively-stained converting enzyme shows the enzyme to have a diameter of about 60 Å. Taking into account the fact that the platinum-carbon shadowing material used to produce freeze-fracture replicas is of finite thickness (about 20 Å), it appears reasonable that replicas of converting enzyme itself would approach 100 Å.

Having been supplied with a homogeneous preparation of hog lung converting enzyme (Dorer et al., 1972), we now have the possibility of localizing the subcellular site of the pulmonary conversion of angiotensin I to angiotensin II by immunocytochemical means.

#### Immunization

We have immunized goats with hog lung converting enzyme using complete Freund's adjuvant. We have on hand liter quantities of an antibody, which by all techniques tested appears to be specific in its reaction with converting enzyme. The antibody has been examined by double immunodiffusion, immunoelectrophoresis and for inhibitory effects on converting enzyme activity.

Graph 1 shows the inhibitory effects of increasing amounts of whole antiserum. The goat which produced this antiserum has been boosted twice more since we obtained these data.

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INHIBITION OF CONVERTING ENZYME

Antiserum (goat) vs.  
hog lung converting enzyme.

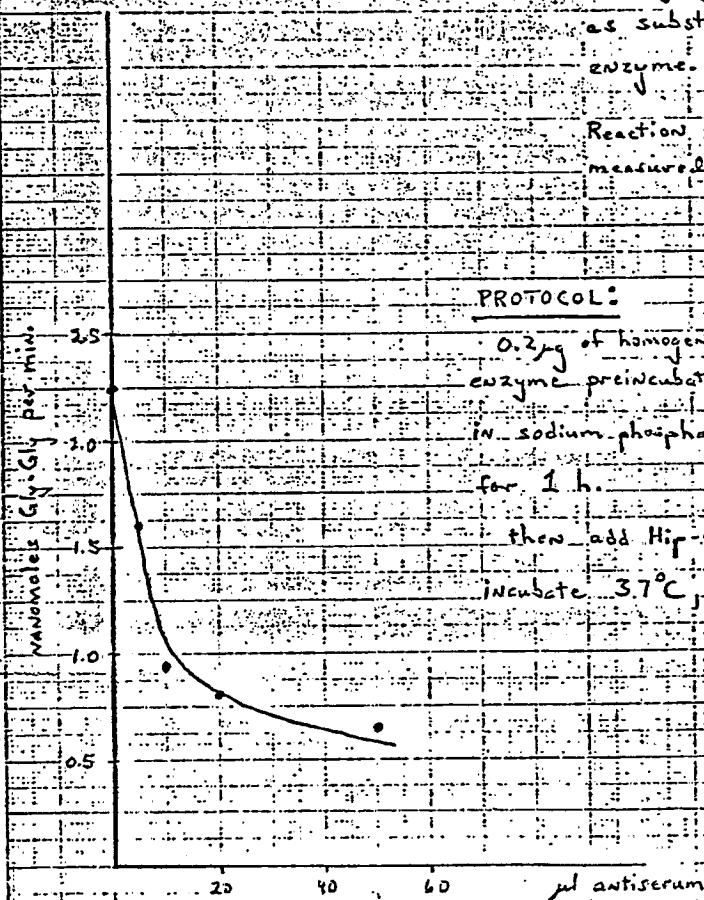
Hippuryl-glycyl-glycine used  
as substrate for converting  
enzyme.

Reaction product: Gly-Gly,  
measured by ninhydrin reaction.

PROTOCOL:

0.2  $\mu$ g of homogeneous lung converting  
enzyme preincubated with antiserum  
in sodium phosphate buffer, pH 8.0  
for 1 h.

then add Hip-Gly-Gly to 1mM,  
incubate 37°C, 1 h.

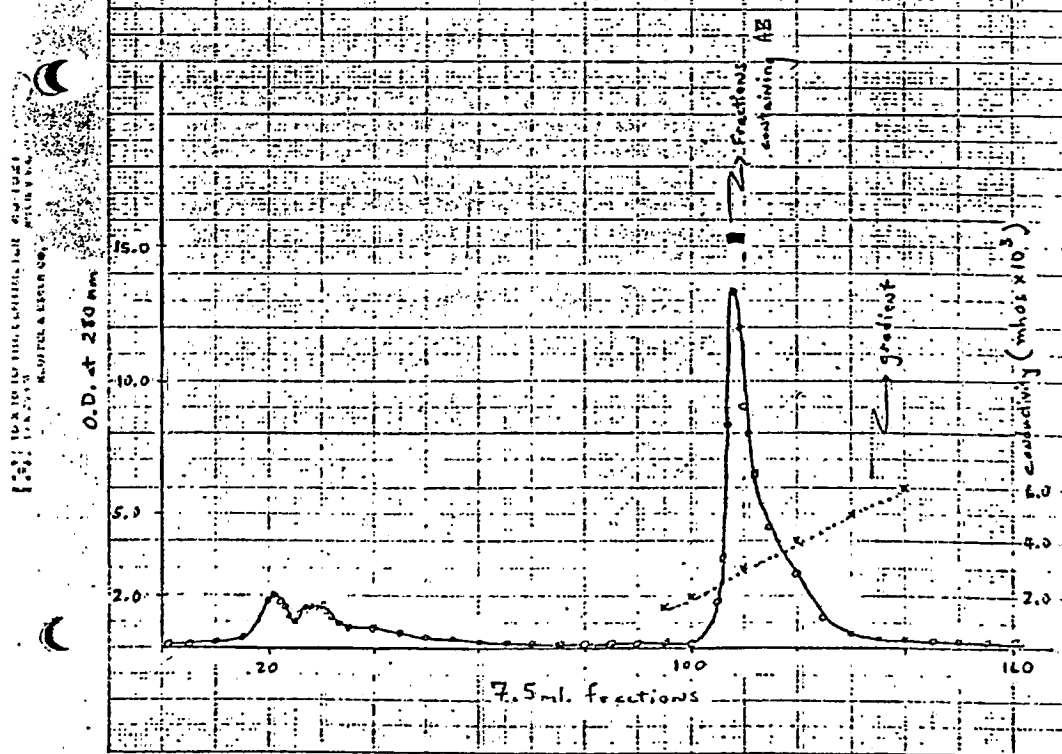


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## PURIFICATION OF ANTIBODY TO HOG LUNG CONVERTING ENZYME

- ① Goat antiserum (50 ml) was treated with  $(\text{NH}_4)_2\text{SO}_4$ , 33% final.
- ② The precipitate was washed with 33%  $(\text{NH}_4)_2\text{SO}_4$  and then was dissolved in 0.15 M NaCl (50 ml).
- ③ The saline-AB solution was dialyzed vs. 5mM sodium phosphate-10mM NaCl, pH 7.5 for 48 h.
- ④ The dialyzed material was applied to a column containing DEAE-cellulose (3x25.2 cm; 0.005M sodium phosphate-0.01M NaCl, pH 7.5; flow rate 20 ml/h). A linear gradient was developed to 0.3M NaCl.



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### Purification of antibody

Much of the time and effort leading up to the localization of converting enzyme by immunocytochemical techniques has focused on the purification of the antibody to eliminate non-specific reactants. The purification procedures are outlined on graph 2. The antibody was precipitated with ammonium sulphate and then chromatographed on DEAE-cellulose using a linear salt gradient. Normal goat serum, for control studies, was processed similarly.

### Conjugation of antibody to marker

We chose to couple antibody to microperoxidase for pilot experiments. Microperoxidase has a major advantage over tracers such as ferritin and horseradish peroxidase in that its monomeric structure is small (MW approx. 2,000). Thus, antibody conjugated to microperoxidase can be readily separated from unconjugated microperoxidase and even from small polymers of microperoxidase that may occur as side products of the conjugation technique.

### Pilot experiments

We have coupled microperoxidase to our purified antibody by glutaraldehyde (Avrameas, 1969, 1970). The conjugate was dialyzed and the non-dialyzable portion was used for cytochemical studies.

Our experiments were modeled on the published techniques of Moriarty and Halmi (1971), Mazurkiewicz and Nakane (1972), and Graham and Karnovsky (1966). We have made progressive modifications, and procedures as they are now conducted in our laboratory are detailed below.

A rat is prepared for perfusion of the lungs as described previously (Smith and Ryan, 1970). The lungs are pumped with Krebs-Henseleit solution until they are freed of blood and then perfused for 10 min with paraformaldehyde-picric acid as fixative (Stefanini et al., 1967).

The paraformaldehyde-picric acid fixed blocks are diced extremely finely under the binocular microscope and then incubated for 30 min in each of the following mixtures. Phosphate buffered saline (PBS) is used as a wash. All incubations are carried out at room temperature on a shaker. All solutions are prepared immediately before use.

- 1) Normal goat serum 1:30 (to cover non-specific sites)  
Wash in PBS
- 2) Converting enzyme Ab/microperoxidase complex, at a dilution of 1:100, but also at dilutions of 1:50 or 1:25
- 3) Diaminobenzidine (DAB)
- 4)  $H_2O_2$  added to DAB for final 1 min
- 5)  $OsO_4$  (1% in veronal acetate buffer) 1 hr  
Dehydration in graded ethanol series  
Embedment in Araldite via propylene oxide

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We have obtained sections which show no impairment of fine structural preservation. The sections are clear and show a fine and precise localization of microperoxidase.

The reaction product is present on much of the luminal plasma membrane but is most prominent on the membranes of caveolae. One striking finding was that endothelium of small vessels, probably small venules, showed a marked localization particularly on the endothelial projections. During the course of our previous ultrastructural studies of pulmonary endothelial cells, we had occasion to examine the luminal surface of the pulmonary artery by scanning electron microscopy. We found that the luminal surface is not flat but is covered by a profuse, irregular array of minute projections (Smith et al., 1971). The function of the projections is not known but it is clear that they vastly increase the surface area of the endothelium and cannot fail to affect flow dynamics at the cell body. Their size and density would produce a meshwork allowing an eddy flow of cell-free plasma over the main body of the cell and we suggested they might have implications in the exchange of metabolites between endothelium and blood. In view of the localization of converting enzyme activity on endothelial projections, we feel it will be useful to determine their role in the metabolism of circulating substances. The prominence of reaction product on projections of venules suggests the possibility that, e.g. the conversion of angiotensin I to angiotensin II occurs immediately after central venous blood is converted to arterial blood. We plan to focus attention on this point.

While it is clear that these pilot experiments require much refinement they serve to show the feasibility of the technique.

#### Conjugation of angiotensin converting enzyme to 8-MP

Our pilot studies have used techniques that might be termed "state of the art." We have come to recognize, as have others, that the "state of the art" techniques require improvement. The major problem centers around the methods of conjugating specific antibody to a marker, e.g. microperoxidase, such that the immunoreactivity and specificity of the antibody are not impaired and the activity of the enzyme is left intact. In fact, the commonly used conjugation techniques, such as those employing glutaraldehyde or carbodiimide, result in the formation of a large number of side products, virtually all of which have the potential of causing artifactual results. For example, in addition to the formation of antibody-marker hybrids, it is likely that antibody-antibody, marker-marker and other complex (straight chain and branched) polymers are formed. Of the desired antibody-marker hybrids, one cannot be sure that each such complex retains immunoreactivity and enzyme activity. The active antibody-inactive enzyme species can be expected to bind antigen, but in a manner so as to be undetectable by electron microscope techniques. The inactive antibody-active enzyme species may yield non-specific electron-dense deposits. In addition, the possibility must be considered that some antibody and some marker molecules do not conjugate but remain in their original states.

Within limits, undesired side products of the conjugation techniques can be eliminated or reduced by separation procedures, e.g. gel filtration.

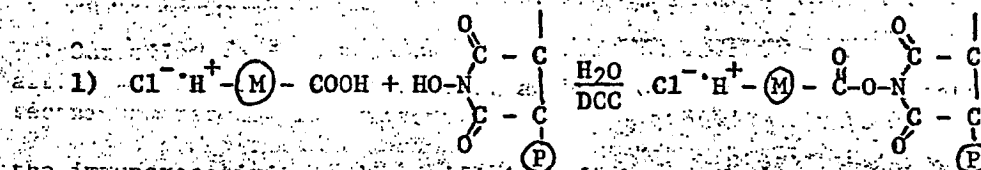
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When immunoabsorption is possible, one can purify active antibody-marker hybrids. However, immunoabsorption is feasible only when antigen is in sufficient supply. Unfortunately, pure angiotensin converting enzyme is in extremely short supply. In any event, immunoabsorption is selective for antibody and not for marker and therefore does not separate active antibody-active enzyme from active antibody-inactive enzyme hybrids, and would not, as a single procedure, eliminate unreacted antibody.

Thus we propose to undertake a program to develop improved, more specific methods of conjugating antibody to microperoxidase (11-MP) or to its lower homolog, the heme-octapeptide (8-MP). It should be emphasized that success using antibody to angiotensin converting enzyme will be immediately translatable for use with other antibodies, including antibodies to nicotine and cotinine. Antibodies to the latter compounds are under development in a parallel program of this laboratory (see Appendix).

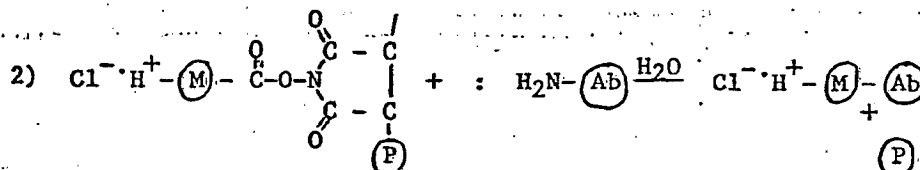
We project two general approaches for improving conjugation techniques. In the first approach, we plan to use a technique developed by a member of this laboratory for solid phase polypeptide synthesis (Laufer, 1968). The marker, 11-MP or 8-MP will be covalently bound to an insoluble N-hydroxy-succinimide polymer (copoly-ethylene-N-hydroxy maleimide).

Legend - P = polymer  
M = marker  
AB = antibody



the immunoreactivity and specificity of the antibody are preserved.

Reaction 1) employs the marker in its hydrochloride form to prevent cyclization. The DCC may lead to polymerization of some of the marker, but these markers can be separated from the derivatized insoluble polymer by filtration. In addition, the hydrochloride will tend to limit polymerization. The desired reaction of marker to the polymer will yield an active ester linkage.



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Reaction 2) effects the conjugation, and leaves the marker-antibody molecule in solution and thus readily separable from the polymer. The only other reactants to be dealt with should be unreacted antibody. The concentration of unreacted antibody can be minimized by using the polymer in excess. The only major problem that we foresee at this time is possible steric hindrance. The antibody constitutes an extremely bulky molecule in comparison with the functional groups of the polymer. Should the problem of steric hindrance arise, we will attempt to develop an alternative scheme using Fab fragments and/or conjugation "bridges" (see below). Our second approach represents a contingency plan: Jamieson and colleagues of the Rockefeller University (Kraehenbuhl et al., 1974) have developed an elegant conjugation scheme but of such complexity that few laboratories could undertake it. In brief, Jamieson's group isolated microperoxidase from the peptic digestion of cytochrome C. The heme-undecapeptide (11-MP) was then reacted with trypsin to remove the N-terminal tripeptide, Val-Gln-Lys. The resulting heme-octaepptide (8-MP) was then isolated from the other reactants by an extensive countercurrent distribution involving over 2,000 transfers. The 8-MP was derivatized after synthesis and purification of the N-hydroxysuccinimide ester of p-formylbenzoic acid. The ester was reacted with the  $\alpha$ -amino group in the presence of pyridine. The activated 8-MP was reacted with antibody to form a Schiff base and the aldehyde group of the derivatized 8-MP was reduced with borohydride. Unreacted 8-MP and unconjugated antibody were removed by gel filtration and ion exchange chromatography (DEAE-cellulose).

Our laboratory is fully staffed and equipped to carry out the techniques described by Jamieson and colleagues. However, we believe it feasible to simplify their techniques, hopefully to a point such that specific conjugations can be carried out by a larger number of laboratories. One development which should make our job easier is that microperoxidase (11-MP) is now available commercially (Sigma). This eliminates the steps required for the isolation of cytochrome C, its degradation with pepsin and isolation of 11-MP itself. Secondly, although we have countercurrent distribution apparatus, we feel that we can simplify the separation of 11-MP from 8-MP by high-voltage electrophoresis, 11-MP being more positively charged because of its lysyl residue. An electrophoretic separation could be effected in a matter of one or two hours versus the week or more required for countercurrent distribution of greater than 2,000 transfers.

Should our first approach (using the insoluble polymer of N-hydroxysuccinimide) work, it will not be necessary to pursue the Jamieson approach. However, should the problem of steric hindrance to reaction of antibody with the polymer prove insuperable, we can combine favorable features of the first approach with those of the second approach; which is to say that we can form an active ester of polymer and p-formylbenzoic acid and then react 8-MP to form the activated 8-MP of the Jamieson procedure. The activation of 8-MP by this scheme should occur spontaneously. By using p-formylbenzoic acid in reaction with the N-hydroxysuccinimide polymer it will not be necessary to isolate the active ester as is necessary in the Jamieson procedure. As in the Jamieson procedure, the p-formylbenzoic acid moiety will "bridge" the antibody to its marker. The remainder of the conjugation to antibody could proceed as described by Jamieson and colleagues.

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The coupling of angiotensin converting enzyme antibody to 8-MP should be of great advantage in our EM localization study. Because of its smaller size (<15 Å diameter), it should result in less alteration of the biological activity of ligands to which it is coupled. In addition, the small size is particularly important for immunocytochemical techniques in which the tagged antibodies are required to penetrate several diffusion barriers such as the plasma membrane and organelle membranes before reacting with antigenic sites. These advantages apply equally to other antibodies, in particular nicotine and cotinine which are being developed in a parallel research program of this laboratory (please see Appendix). This was a preliminary study of the scheme using tag fragments and/or conjugation "bridges" (see 1974).

Much of the chemistry required for our proposed studies is applicable for conjugation of nicotine or cotinine themselves (in addition to their antibodies) to microperoxidase (either 11-MP or 8-MP) for purposes of examining their fates by EM techniques. While a strong a priori argument can be made that nicotine or cotinine labelled intrinsically with tritium is more desirable than nicotine labelled extrinsically (as with 8-MP), the fact of the matter is that the former must be used in autoradiography whereas the latter can be used cytochemically. Cytochemical reactions almost invariably result in finer depositions at the level of electron microscopy. In addition, MP (the extrinsic label) is an enzyme and therefore provides through its activity a great amplification factor per molecule of nicotine and should greatly improve the opportunities for localizing small quantities of the drug in tissues prepared for EM.

## 2. Development of rapid, simple and sensitive assays of angiotensin converting enzyme (kininase II)

As stated above, it is now established that one of the pulmonary enzymes that degrades bradykinin (kininase II) is the same enzyme that converts angiotensin I to its active lower homolog, angiotensin II (angiotensinase converting enzyme). However, for the purposes of convenient discussion, the enzyme will be called converting enzyme. The point that we wish to emphasize is that the development of a rapid, simple and sensitive assay for studying the conversion of angiotensin I will yield data of equal importance to the inactivation of bradykinin.

Converting enzyme is a dipeptide carboxypeptidase of relatively little specificity. In addition to its ability to metabolize angiotensin I and bradykinin, it will also release the C-terminal dipeptides of hippuryl (Hip)-His-Leu, Hip-Gly-Gly, Z-Phe-His-Leu and the B-chain of insulin (for review, see Bakhle, 1974).

In previous studies on the purification of lung converting enzyme, Cushman and Cheung (1969) developed a synthetic substrate, Hip-His-Leu, that is resistant to non-specific "angiotensinase" enzymes while being highly reactive with converting enzyme. The substrate has the further advantage that the hippuric acid moiety is soluble in ethyl acetate while the substrate itself and the histidyl-leucine moiety are not. We have been especially attracted to the use of Hip-His-Leu because of the simplicity of solvent partitioning as a separation technique. However, the assay devised by Cushman and Cheung is not very sensitive: In their protocol, hippuric acid, released as a consequence of reaction of Hip-His-Leu with converting enzyme, is quantified by spectrophotometry (absorption at 218 nm).

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We propose to increase the sensitivity of the assay by labelling the hippuric acid moiety. Specifically,  $^{14}\text{C}$ -glycine will be coupled to benzyl chloride to yield hippuric acid. The  $^{14}\text{C}$ -hippuric acid will, in turn, be coupled to histidyl-leucine by carbodiimide condensation. We have synthesized the starting material, t-Boc-His-Leu-benzyl ester. After deprotection of the t-Boc- and after carbodiimide condensation, the benzyl ester will be removed by anhydrous HF or by saponification. These advantages apply equally to the assay. We envisage an assay protocol only slightly modified from that of Cushman and Cheung. After reaction of substrate with dispersed cells, the incubation medium will be acidified and extracted with ethyl acetate. The ethyl acetate layer (containing  $^{14}\text{C}$ -hippuric acid) can be incorporated directly in scintillation fluid and counted.

The proposed assay should be sufficiently simple so as to allow essentially continuous measurements of the activity of converting enzyme in situ. For example, isolated lungs, prepared for single circulation experiments, can be cannulated via the left ventricle such that the pulmonary venous effluent is directed to a fraction collector. The test tubes of the fraction collector can be pre-loaded with an acid-ethyl acetate mixture to facilitate immediate separation of the hippuric acid moiety from undegraded Hip-His-Leu and from the second reaction product, His-Leu.

Thus it should be possible to subject the lungs to a variety of stimuli (e.g. under inflation, hyperinflation, hypoxia, hyperoxia, etc.) while measuring changes in the "flux rate" of the conversion of angiotensin I (or the degradation of bradykinin) at minute intervals. We use the term, "flux rate," as a general one to signify net changes in metabolism of the Hip-His-Leu substrate by whole lungs. Clearly, changes in the activity of converting enzyme may play only a minor role in these net changes; vasomotion, redistribution of blood flow, blood pressure, flow rates, etc., being much more important. However, for the concept of "endocrine" functions, the net changes are of paramount importance, as these are the changes which should determine how much angiotensin I, angiotensin II and bradykinin will enter the systemic arterial circulation and thus be supplied to target organs.

#### Significance

The ability of the lungs to process selectively certain circulating vasoactive substances has only recently come to light. In addition to the importance of these non-ventilatory functions to the lungs themselves, the conversion of angiotensin I to angiotensin II illustrates the ability of the lungs to produce substances which may influence organs at a distance. In this respect the lung is acting as an endocrine organ.

As mentioned previously it was evident from the beginning of our studies that we had discovered an entirely new mechanism of hormone metabolism. Nevertheless, there is little information on the effects of physiologic stimuli or environmental inhalants on these newly recognized functions or of the effects of failure by the lungs to carry out these functions.

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We propose to increase the sensitivity of the assay by measuring the hippuric acid moiety. Specifically, 14C-labeled substrates will be used. Consequently, we have outlined a program for the definitive subcellular localization of angiotensin converting enzyme. In addition we plan to develop rapid flux assay methods for the detection of specific metabolites. While the conceptual and technical aspects of the program are ambitious, they present a rare opportunity. Success in the technical development of these studies will form the basis for determining the effects of drugs such as nicotine and of inhalants on a specific endocrine function of the lungs.

After 24 hours, after addition of substrate with appropriate buffer, the incubation medium will be acidified and extracted with ethyl acetate. The ethyl acetate layer (containing the 14C-labeled substrate) will be dried and the radioactivity determined by scintillation spectrometry.

The proposed study should be sufficiently simple to be carried out by a research assistant. The activity of converting enzyme will be measured by the amount of 14C-labeled substrate converted to the product.

The results of the study will be compared with the results of the control group.

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**APPENDIX: CONJUGATION OF NICOTINE, COTININE AND THEIR ANTIBODIES TO  
MARKERS FOR ELECTRON MICROSCOPY**

**Reference:** See correspondence from Dr. R.C. Hockett to Dr. A. Castro,  
2/15/74 (last paragraph) and from Dr. Castro to Dr. Hockett,  
2/28/74.

Whereas a major aim of the research proposed in this application is to develop specific conjugation techniques for antibody to lung angiotensin converting enzyme, we wish to emphasize that the techniques to be developed should be immediately applicable for use with other antibodies and other antigens (e.g. nicotine, cotinine and their antibodies). In addition, we would like to point out that our laboratory has or will have on hand all of the necessary starting materials. Through a parallel research program (Nicotine in Blood: Detection by Radioimmunoassay), our staff have prepared 6-amino nicotine. This derivative was used for conjugation to bovine serum albumin (BSA) for immunization purposes and has also been used to prepare cotinine in a form suitable for conjugation to BSA. If we meet our immunization schedules, antibody to nicotine should be available by January 1, 1975, and antibody to cotinine should be available by May 1, 1975. These estimated dates of availability fit well with our schedule for developing new, specific techniques for conjugating antibody to the heme-octapeptide form of microperoxidase (8-MP).

We have a substantial quantity of 6-amino nicotine on hand. This derivative lends itself immediately to conjugation to microperoxidase using the N-hydroxysuccinimide polymer reacted with p-formylbenzoic acid (see text of this application). A nicotine-8-MP conjugate should be of considerable usefulness in studies of nicotine receptors at the level of electron microscopy and should supplement parallel studies using <sup>3</sup>H-nicotine in autoradiography.

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Stefanini, M., De Martino, C. and Zamboni, L. Nature. 216:174, 1967.

Vane, J.R. Br. J. Pharmac. 35:209, 1969.

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SEITE 1. RUMPT 1. HIC  
Space and facilities available (continued)

Equipment available by appointment (located in main Papanicolaou Building)

Philips EM 200 electron microscope

Steier Balzer's freeze-etch apparatus (about 1970-1971)

AMR SEM 900 scanning electron microscope

Van der J. K. H. J. P. 25:20, 1968

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CURRICULUM VITAE

Name: Una Scully Ryan

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Education:

Bristol University, Bristol, England 1960 - 1963

B.Sc. (Zoology - Special Honors, First Class)

B.Sc. (Chemistry - Subsidiary, First Class)

B.Sc. (Microbiology - Subsidiary, First Class)

Cambridge University, Cambridge, England 1968

Ph.D. (Cell Biology)

Research and/or Professional Experience:

1963 - 1967 Research Student, Department of Zoology, University of Cambridge, England (On leave of absence, 1964-1966)

1964 - 1966 Predoctoral Fellow, Department of Biology, University of Virginia, Charlottesville, Virginia

1966 - 1967 Predoctoral Fellow, Department of Medicine, University of Miami, Miami, Florida

1967 - 1971 Visiting Investigator, Laboratories for Cardiovascular Research, Howard Hughes Medical Institute, Miami, Florida

1970 - 1971 Director, Laboratory for Ultrastructure Studies, Howard Hughes Medical Institute, Miami, Florida

1967 - 1972 Instructor in Medicine, Department of Medicine, University of Miami School of Medicine, Miami, Florida

1968 - present Adjunct Assistant Professor of Biology, Department of Biology, University of Miami, Miami, Florida

1971 - present Assistant Professor of Medicine, Department of Medicine, University of Miami School of Medicine, Miami, Florida

1972 - present Senior Scientist, Papanicolaou Cancer Research Institute, Miami, Florida

Academic and Professional Honors:

B.Sc. with First Class honors in Zoology, Chemistry and Microbiology  
Bristol University Open Exhibition, 1960  
U.K. State Scholarship, 1960  
County Major Scholarship, 1960  
D.S.I.R. Research Fellowship, 1963  
Ethel Sargent Research Fellowship (awarded twice), 1964 and 1965  
Science Research Council Research Fellowship, 1966  
Established Investigator, American Heart Association, 1972 - present  
Member, Pulmonary Diseases Advisory Committee,  
National Heart and Lung Institute

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- 2 -

Una S. Ryan

Societies:

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Cambridge University, Cambridge, England

1968

1968 - 1971

Professor of Biology, University of Miami

Biology, University of Miami, Miami, Florida

1971 - present

Associate Professor of Biology, Department of Biology

1972

Senior Lecturer, Department of Biology, University of Miami

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- 7) Smith, U.: Colour change in the stick insect. Ph.D. Thesis, Cambridge, University, 1967.
- 8) Martelo, O.J., Manyan, D.P., Smith, U. and Yunis, A.A.: Chloramphenicol and bone marrow mitochondria. J. Lab. Clin. Med., 74:927, 1969.
- 9) Thompson, A., Halbert, S.P. and Smith U.: The toxicity of streptolysin O for beating mammalian heart cells in tissue culture. J. Exp. Med., 131:745, 1970.
- 10) Smith, U.: Some aspects of the fine structure and function of the sub-commissural organ of the embryonic chick. Tissue & Cell, 2:19, 1970.
- 11) Smith, U. and Ryan, J.W.: An electron microscopic study of the vascular endothelium as a site for bradykinin and adenosine-5'-triphosphate inactivation in rat lung. In Vol. 8, Adv. Exp. Med. Biol. (ed. N. Back, F. Sicuteri and M. Rocha e Silva), Plenum Press, N.Y., 1970, pp. 249-262.
- 12) Smith, U., Smith, D.S. and Yunis, A.A.: Chloramphenicol-related changes in mitochondrial ultrastructure. J. Cell Sci., 7:501, 1970.
- 13) Yunis, A.A., Smith, U. and Restrepo, A.: Reversible bone marrow suppression from chloramphenicol. Arch. Int. Med., 126:272, 1970.
- 14) Smith, U. and Ribbons, D.W.: The fine structure of Methanomonas methanoxidans. Archiv. fur Mikrobiologie, 74:116, 1970.

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- 29) Smith, U., Ryan, J.W. and Smith, D.S.: Freeze-etch studies of the plasma membrane of pulmonary endothelial cells. *J. Cell Biol.*, 56:492, 1973.
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- 14) Ryan, J.W. and Smith, U.: Metabolism of angiotensin I by endothelial cells. Proc. XXth Colloquium-Protides of the Biological Fluids, Brugge, May 3-7, 1972.
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- 17) Hughes, G.M., Ryan, J.W. and Smith, U.: Freeze-fractured lamellate bodies of Protopterus lung: A comparative study. Proc. Physio. Soc., Cork, Sept. 14-15, 1973.

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## CURRICULUM VITAE

Name: James W. Ryan

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Education: Dartmouth College, 9/53-6/57, A.B. Political Science  
Cornell University Medical College 9/57-6/61, M.D.  
Oxford University, 9/65-8/67, D.Phil., Biochemistry

Positions Held:

| <u>Position</u>  | <u>Superior</u>                                   | <u>Institution</u>   | <u>Dates</u>      |
|--|---|--|-------------------|
| Interne  |   | Montreal General Hospital<br>McGill University                       | 6/61 -<br>6/62    |
| Assistant Resident, Medicine   | Professor D. G. Cameron                           | As above   | 7/62 -<br>7/63    |
| Research Associate   | Dr. Seymour Kety                                  | National Institute of Mental Health<br>National Institutes of Health | 7/63 -<br>9/65    |
| Guest Investigator, Assistant Professor of Biochemistry (from April, 1968) | Professor R. B. Merrifield<br>Dr. John M. Stewart | Rockefeller University<br>New York, New York                         | 9/67 -<br>9/68    |
| Associate Professor of Medicine  |   | University of Miami<br>Miami, Florida                                | 9/68 -<br>present |
| Senior Scientist   |   | Papanicolaou Cancer Research Institute<br>Miami, Florida             | 9/72<br>present   |

Academic and Professional Honors:

Regional Scholarship - Dartmouth College, 1953-1957  
Regional Scholarship - Cornell University Medical College, 1957-1961  
Summer Research Traineeships, U.S.P.H.S., 1958-1959 - Cornell University Medical College  
Smith, Kline and French Travelling Fellowship, 1960  
William Mecklenberg Polk Research Prize, 1961 - Cornell University Medical College  
Training award for studies in biological psychiatry, 1960-1961, Cornell University Medical College  
Travel award, Rockefeller Foundation, 1962  
Research Prize, Montreal Clinical Society, 1963  
Postdoctoral Fellow, U.S.P.H.S., 1965-1967  
Honorary Medical Officer to the Regius Professor of Medicine, Oxford, 1965-1967

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Academic and Professional Honors (Cont'd.)

William Waldorf Astor Travelling Fellowship, 1966  
 Special Fellowship, U.S.P.H.S., 1967 - 1968  
 Career Development Award, U.S.P.H.S., March, 1968-September, 1968  
 Investigator, Howard Hughes Medical Institute, 1968-1971  
 Pfizer Travelling Fellow, University of Montreal, October, 1972  
 Visiting Professor, Clinical Research Institute of Montreal, January - June, 1974

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Research Projects and Appointments:

- 1962 - 65 The rôle of bradykinin in the pathogenesis of acute pancreatitis.  
 Supervisor: Dr. Alan G. Thompson, Montreal General Hospital,  
 Montreal, Quebec.  
 Position: Research Associate.
- 1963 - 65 Studies of serum factors in schizophrenia.  
 Supervisor: Doctors Seymour Kety and Jack Durell,  
 Clinical Center, National Institutes of Health, Bethesda, Maryland.  
 Position: Research Associate.
- 1965 - 67 Studies of renin in plasma and in solid tissues other than the kidney.  
 Supervisor: Professor Sir George W. Pickering, FRS,  
 University of Oxford, Oxford, England.  
 Position: Honorary Medical Officer to the Regius Professor of Medicine.
- 1967 - 68 Metabolism of vasoactive polypeptides.  
 Supervisor: Dr. John M. Stewart, Rockefeller University, New York.  
 Position: Guest Investigator, September, 1967 - April, 1968;  
 Assistant Professor, April, 1968 - September, 1968.
- 1968 - 1) Metabolism of vasoactive polypeptides;  
 2) Non-ventilatory, endocrine functions of the lungs;  
 3) Renal hypertension. Howard Hughes Medical Institute,  
 Investigator (1968-1971);  
 Associate Professor of Medicine, University  
 of Miami School of Medicine (1968 - ) and  
 Senior Scientist, Papanicolaou Cancer Research  
 Institute

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Publications:

- 1) Diniz, C.R., Carvalho, I., Ryan, J.W. and Rocha e Silva, M.: Micromethod for the determination of bradykininogen in blood plasma. *Nature*, 192:1194, 1961.
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- 3) Ryan, J.W., Moffat, J.G. and Thompson, A.G.: Role of bradykinin in the development of acute pancreatitis. *Nature*, 204:1212, 1964.
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- 5) Ryan, J.W., Brown, J.D. and Durell, J.: Antibodies affecting metabolism of chicken erythrocytes: Examination of schizophrenic and other subjects. *Science*, 151:1408, 1966.
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- 7) McKenzie, J.K., Ryan, J.W. and Lee, M.R.: Effect of laparotomy on plasma renin activity in the rabbit. *Nature*, 215:542, 1967.
- 8) Ryan, J.W. and Ferris, T.F.: Release of a renin-like enzyme from the pregnant uterus of the rabbit. *Biochem. J.*, 105:16C, 1967.
- 9) Ryan, J.W.: A rapid, simple method for the assay of renin in rabbit plasma. D. Phil. Thesis, Univ. of Oxford, 1967.
- 10) Ryan, J.W.: Renin-like enzyme in the adrenal gland. *Science*, 158:1589, 1967.
- 11) Ryan, J.W., Steinberg, H.R., Green, R., Brown, J.D. and Durell, J.: Controlled study of the effects of plasma of schizophrenic and non-schizophrenic psychiatric patients on chicken erythrocytes. *J. Psychiat. Res.*, 6:33, 1968.
- 12) Ryan, J.W., Brown, J.D. and Durell, J.: Concordance between two methods of assaying plasma effects on chicken erythrocyte metabolism. *J. Psychiat. Res.*, 6:45, 1968.
- 13) Ryan, J.W., McKenzie, J.K. and Lee, M.R.: A rapid, simple method for the assay of renin in rabbit plasma. *Biochem. J.*, 108:679, 1968.
- 14) Ryan, J.W. and McKenzie, J.K.: Properties of renin substrate in plasma with a note on its assay. *Biochem. J.*, 108:687, 1968.

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Publications (Continued):

- 15) Johnson, D.C. and Ryan, J.W.: Degradation of angiotensin II by a carboxypeptidase of rabbit liver. *Biochim. Biophys. Acta*, 160:196, 1968.
- 16) Ryan, J.W., Roblero, J. and Stewart, J.M.: Inactivation of bradykinin in the pulmonary circulation. *Biochem. J.*, 110:795, 1968.
- 17) Ryan, J.W. and Johnson, D.C.: The renin-like enzyme of rabbit uterus. *Biochim. Biophys. Acta*, 191:386, 1969.
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## CURRICULUM VITAE

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Research and/or Professional Experience:

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| 1968-1970    | Honors Program in chemistry, Olivet College, Olivet, Mich.  |
| 1968-1970    | Chemistry Laboratory Instructor, Olivet College, Olivet, Mich.  |
| 1970-1974    | Graduate student research in autonomic and endocrine pharmacology, University of Virginia, Charlottesville, Va.               |
| 1972-1973    | Lecturer in pharmacology, Master and Practitioner Programs for Nursing Students, University of Virginia, Charlottesville, Va. |
| 1973-present | Postdoctoral Fellow, Papanicolaou Cancer Research Institute, Miami, Fla.  |

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1970

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Publications:

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